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14. ABSTRACT

Stat3 is a transcription factor constitutively active in a large number of breast cancers and other tumors, where it works as a central player in the activation of multiple oncogenic pathways. We developed a method to modulate endogenous Stat3 alternative splicing using modified antisense oligonucleotides, to induce the dominant negative Stat3-beta variant in vitro and in vivo. Switching from the full length Stat3 isoform to the stat3-beta variant leads to increased tumor cell death and complete tumor regression in animal models via a novel modulatory mechanism.

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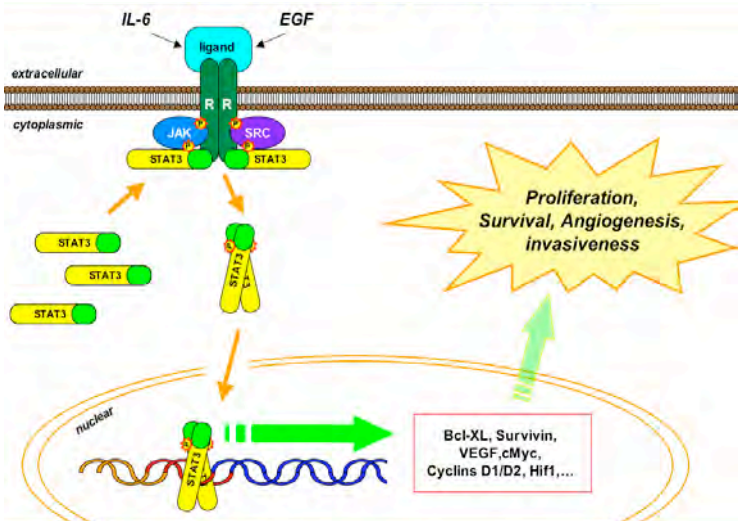
INTRODUCTION

Signal transducer and activator of transcription (STAT) proteins are a family of transcription factors implicated in growth factors and cytokines signalling. In the canonical model of STATs signalling, singles monomers are normally sequestered in the cytoplasm in an inactive form. Their activation is initiated by tyrosine phosphorylation, usually mediated by the binding of cytokines or growth factors to their membrane receptors and/or by intracellular oncogenic tyrosine kinases, such as JAKs and Src. Upon cytoplasmic tyrosine phosphorylation, two STAT monomers dimerize, translocate to the nucleus and bind to specific promoter sequences, thereby regulating gene expression (Figure 1) [1]. Under physiological conditions, every STAT protein has a limited activation period that typically lasts from few minutes to several hours, while persistent activation of STAT proteins, in particular STAT3 and STAT5, is observed in a wide variety of cancers[2]. In recent years a body of works has expanded the standard STAT signalling mode: unphosphorilated STAT homodimers can also be found in the cytoplasm [3, 4], they can shuttle between the cytoplasm and the nucleus [5, 6] and once inside the nucleus they can function as transcription factors [7-10].

STAT3 is implicated in a vast range of physiological processes including cellular proliferation, differentiation, inflammation and immune response [11-14]. STAT3 can also act as an oncogene, able to induce cellular transformation and tumorigenesis[15] and its constitutive activation has been reported in nearly 70% of haematological and solid tumors[16]. Because of its wide range of functions, STAT3 is involved in many aspects of carcinogenesis such as proliferation, survival, angiogenesis and metastasis[17, 18] and can also contributes to tumor escape from natural immune surveillance[19, 20].

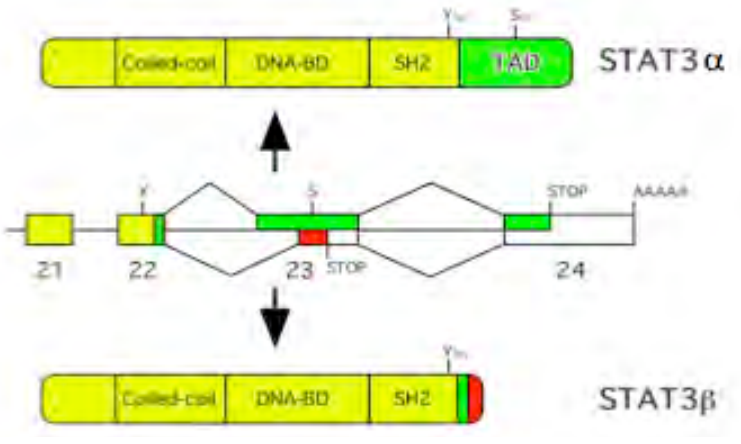
In addition to its oncogenic nuclear transcriptional functions, STAT3 has been shown have a role in tumorigenesis independent of its tyrosine-phosphorylation status and nuclear localization. In fact, STAT3 interaction with a microtubule (MT)-destabilizing protein in the cytoplasm [21, 22] has been shown to have a direct effect on cell-motility, whereas mitochondrial STAT3 has supports Ras-dependent malignant transformation [14, 23].

Because of its pivotal position at the convergence of many oncogenic tyrosin-kinase signalling pathways, STAT3 seems to



1. Stat3 signaling. *Upon cytokine or growth factor receptor binding, Cyoplasmic Stat3 gets recruited to the plasma membrane and activated via tyrosine phosphorylation by Receptor Tyrosine Kinases (RTKs, such as PDGFR or EGFR), or by non-RTKs (such as Src and JAK). it dimerizes and is translocated to the nucleus where it binds DNA to activate its target genes. Stat3 is tightly regulated by phosphatases, SOCS (suppressor of cytokine signaling) and PIAS (protein inhibitor of activated Stats) and participates into a very complex network of signaling interactions that includes other STAT proteins, additional transcription factors and regulators (not shown).*

be particularly suitable as a molecular target for cancer therapy, especially considering that tumor cells tend to become dependent on persistent STAT3 signalling and are more sensitive to its inhibition than normal cells[16, 24]. Indeed, direct inhibition of STAT3 activities by multiple means, such as dominant negative overexpression[25], antisense oligonucleotides[26], RNAi[27, 28] or small drug inhibitors[29] results in growth inhibition and induces apoptosis in various model systems[30].



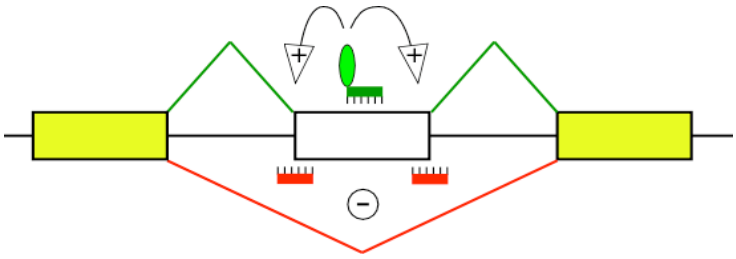
2. Stat3 alternative splicing. Schematic representation of STAT3 exon 23 alternative splicing: alternative acceptor site usage in exon 23 generates both the full length STAT3α and the truncated variant STAT3β that lacks the transactivation domain (TAD) substituted by a unique tail of seven amino acids (shown in red). A phospho-tyrosine residue (Y705), key for dimerization and activity, is retained, whereas phospho-serine 727, also involved in modulating stat3 activity is only present in the alpha variant.

STAT3 exists in two main isoforms: the full-length STAT3α and the truncated STAT3β, generated by the alternative splicing of exon 23 (Figure 2) [31]. STAT3β lacks the protein's 55 C-terminal residues, including STAT3α transactivation domain (TAD), replaced by a tail of seven unique amino acids. STAT3β can be efficiently phosphorylated at Y705 but it lacks S727, whose phosphorylation has been shown to stimulate transcriptional activity and to trigger prolonged nuclear retention [32, 33]. Nevertheless STAT3β can still heterodimerize with itself or the full-length STAT3α and translocate to the nucleus and it was originally thought to inhibit gene transactivation, thus interfering with STAT3 signalling[34]. Indeed, STAT3β has been described as a dominant negative regulator of transcription and its overexpression has been shown to induce apoptosis and to inhibit tumor growth[25, 35]. However, STAT3β novel functions have been recently described[33, 36] and distinctive properties are beginning to emerge[37]. In particular, mice lacking only STAT3α are able to rescue the embrionic lethality of STAT3 null mice thus implying that STAT3β is not a dominant negative factor[38]. Interestingly, mice lacking only STAT3β have no apparent developmental abnormalities; however, when challenged with endotoxins, they exhibit impaired recovery and upregulation of a subset of inflammation-related genes, pointing to the involvement of STAT3β in the control of the inflammation response[39]. This raises the possibility that STAT3β might not just be a truncated dominant negative version of STAT3α, but a protein also capable of modulating its own set of selective target genes, performing unique biological functions and, in doing so, actively contributing to the complex biology of STAT3.

Alternative splicing (AS) is a highly regulated process that generates functionally diverse protein isoforms, accounting for much of the complexity of the human genome, as over 75% of all human genes undergo alternative splicing[40, 41]. Aberrant alternative splicing leads to inappropriate expression levels of

otherwise correct splice variants by shifting their splicing patterns[42]. This is particularly evident in cancer cells, with the appearance of cancer-associated splicing isoforms in hundreds of different genes[43]. Although for a lot of these isoforms a definite cause-effect relationship remains to be established, it is clear that the expression of specific splice variants of many cancer-related genes confers a growth advantage and plays a determinative role in the tumorigenic process[44-46]. Cancer-associated splice variants can contribute to tumorigenesis in different ways, as they are involved in the control of cell signaling, cell proliferation, DNA repair, angiogenesis and extracellular interactions[47, 48].

Redirection of alternative splicing through the use of antisense oligonucleotides that induce specific and persistent splicing switching potentially constitutes a powerful therapeutic approach, with great potential both in vitro and in vivo[49-51]. In fact, splicing redirection shares with other antisense-based technologies (such as RNAi or RNAseH-dependent targeting) the high degree of target specificity but it is unique in its ability not only to knock down the desired target, but simultaneously to induce the expression of a more favourable isoform.



3. Antisiense-based redirection of alternative splicingg. *Positive strategy (top): chimeric antisense compounds promote specific splicing events independent of pre-existing cis-elements. Negative strategy (bottom): compounds compete with factors recognizing existing cis-elements, exerting the opposite effect on splice site selection. If the cis-elements are silencers, the overall effect is positive*.

In the funded study we have developed morpholino-based oligonucleotides able to redirect endogenous STAT3 alternative splicing from the predominant oncogenic STAT3α isoform, to the underrepresented STAT3β variant. This redirection is associated to increased cell-death in Stat3-dependent cells *in vitro* and with complete tumor regression *in vivo.* Interetingly, full knock-down of STAT3 was not as effective as the splicing switch in promoting cell death and tumor regression, suggesting that STAT3β may induce cell death by a mechanism different than a straight dominant negative effect. Indeed, we found that the alpha-to-beta switch activates a specific transcription program that includes downregulation of survival factors, including LEDGF, PCAF, Cyclin C and Stat1beta. Therefore, our results not only validate STAT3β role as a potent anti-tumorigenic molecule, but also highlight STAT3β's ability to exert its effects through the modulation of a novel specific set of target genes, which themselves might represent novel targets for cancer therapy.

BODY

Deletion analysis of STAT3 exon 23

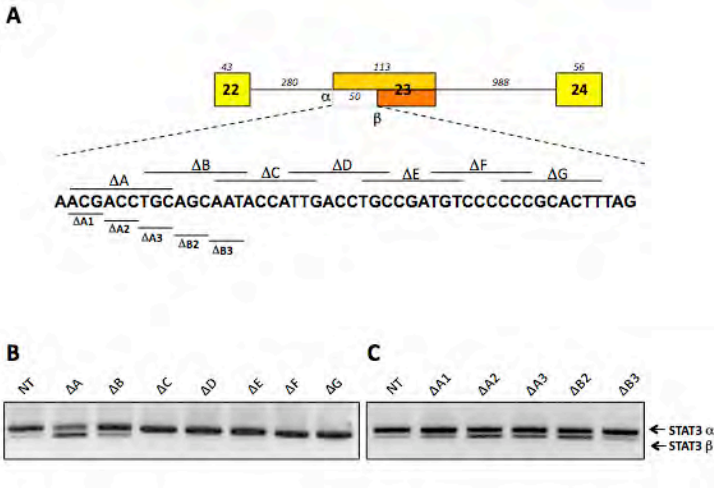
The naturally occurring isoform STAT3b is generated by the use of an alternative acceptor site within exon 23. This causes an internal deletion spanning the first 50 nucleotides of exon 23 that results in a frameshift introducing seven alternative amino acids followed by a stop codon (Figure 2). The resulting protein is therefore a truncated version of STAT3 lacking the C-terminal transactivation domain (TAD).

Although the position of the two alternative acceptor sites from exon 23 of STAT3 has been identified, nothing is known about the regulation of this AS event[52].

We therefore set up to identify the regulatory cis-elements that modulate exon 23 alternative splicing. We first generated a STAT3 minigene containing exons 22-23-24 and the intervening introns. When this minigene is transiently transfected into HeLa cells, its splicing pattern reproduces the relative levels of STAT3a and STAT3b observed in the endogenous STAT3 gene, as assayed by RT-PCR, suggesting that no essential regulatory elements were lost in the subcloning steps (Supplemental Figure1A).

The minigene system was then used to perform a systematic small deletion analysis of exon 23 and surrounding introns in order to identify regulatory cis-elements. A first set of seven partially overlapping 9bp deletions (DA to DG) was introduced in the first 50nt of exon 23 (a specific region), from position +2 to +47 (Figure 4A). Each deletion mutant was transiently transfected into HeLa cells, and STAT3a/STAT3b ratios were monitored by RT-PCR. Interestingly, deletions DA and DB induced a significant switch from the STAT3a to STAT3b isoform, suggesting the presence of a putative Exonic Splicing Enhancer (ESE) (Figure 4B), as predicted also by the ESE-finding algorithms ESE finder and PESX (Supplementary Figure 1B). Smaller 3bp deletions encompassing DA and DB restricted the putative ESE from position +2 to +12 (Figure 4C).

In order to analyze the remaining sequence of exon 23, which encompasses the a and b common region, a second STAT3 minigene was generated. The weak pyrimidine tract responsible for the STAT3b 3'



4. Identification of STAT3 exon23 *cis*-elements. (A) (Top) Diagram of STAT3 minigene: exon and intron sizes are indicated (nt). (Bottom) Lines above and below exon 23 sequence indicate the exact position of the 9nt (DA to DG) and 3nt (DA1 to DB3) deletions generated.

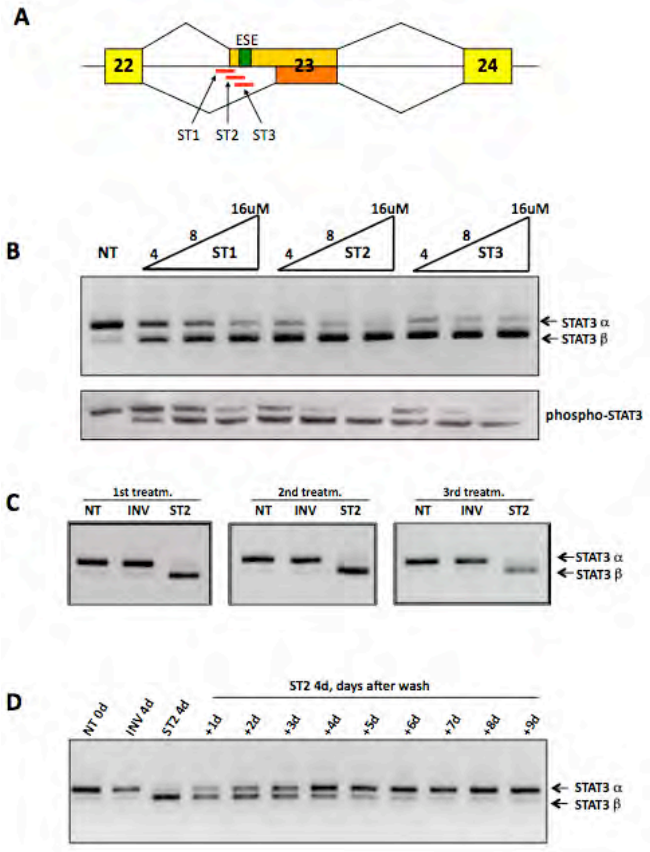
(B, **C**) RT-PCR analysis of STAT3 a/b levels in HeLa cells transfected with the 9nt (DA to DG) and the 3nt (DA1 to DB3), respectively, exon 23 deletion mutants. NT, untreated.

splice site recognition was strengthened by a double mutation; this resulted in a significant induction of STAT3b splicing (Supplemental Figure 1C). A first set of six partially overlapping 12bp deletions (DH to DO) were introduced from position +52 to +109 (Supplemental Figure 1D). Upon transient transfection into HeLa cells, we noticed that deletion DH and, to a lesser extent deletion DK, caused the more dramatic changes in the STAT3 a/b ratio suggesting the presence of regulatory sequences in this region (Supplemental Figure 1E). Testing of additional mutants containing smaller 3bp deletions encompassing DH and DK pointed to nucleotides in positions from +52 to +54 of exon 23 (deletion DH1) as the more likely to contain a regulatory element (Supplemental Figure 1F). Any of the 60bp overlapping deletions in the surrounding introns resulted in obvious changes in STAT3 splicing (data not shown).

Modulation of STAT3 exon 23 alternative splicing

The identification of a putative ESE at the 5' end of exon 23 of the STAT3 gene prompted us to design three different morpholino oligonucleotides directed at blocking either the alpha 3' splice site (ST1) or the putative ESE (ST3) or both of them (ST2) (Figure 5A). An oligonucleotide (INV) containing a scramble sequence was used as a control in all experiments.

MDA-MB-435s breast carcinoma cells were treated with increasing concentration (4μM, 8μM and 16μM) of ST1, ST2 and ST3 morpholinos using Endo-porter as delivery reagent. After 4 days, efficacy of the treatments was monitored by quantification of endogenous STAT3a/b ratios both at the RNA (RT-PCR) and protein (Western blot) levels (Figure 5B). All three oligonucleotides, ST2 and ST3 in particular, induced a dramatic switch from STAT3a to STAT3b. The ST2 morpholino was chosen for all subsequent STAT3 exon 23 splicing redirection experiments since its sequence is 100% homologous to that of mouse exon 23, and can therefore be used both in



5. Modulation of STAT3 alternative splicing

(A) Schematic localization of the morpholino oligos relative to exon 23 a 3' splice site and putative ESE. **(B)** RT-PCR and Western blot analysis of STAT3 a/b levels in MDA-MB-435s cells treated with increasing concentration of ST1, ST2 and ST3 morpholino oligos for 4 days. (**C**) RT-PCR analysis of STAT3 a/b levels in MDA-MB-435s cells treated with either the inverted (INV) or ST2 morpholino oligos for 3 consecutive treatments of 4 days each. **(D)** RT-PCR analysis of STAT3 a/b levels in MDA-MB-435s cells pre-treated with either INV or ST2 morpholino oligos for 4 days and then let grown in absence of treatment for up to 9 days. NT, untreated.

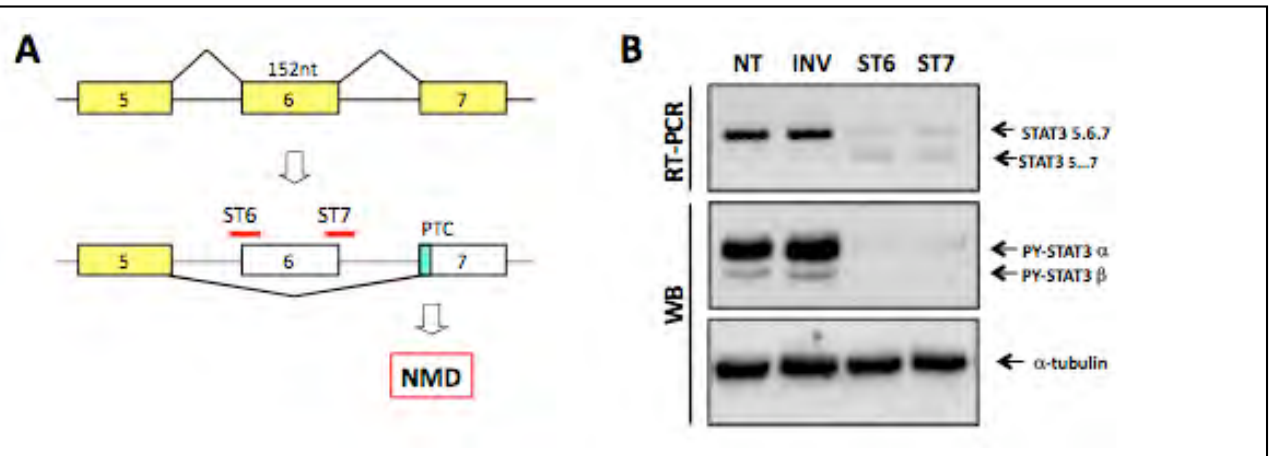
human and mouse cells.

To investigate whether the observed splicing switch is maintained over time, we treated MDA-MB-435s cells with the ST2 morpholino for 4 days, trypsinized, plated and re-treated them for two additional rounds, for a total of 12 days. Analysis of the RNA levels of STAT3a and STAT3b by RT-PCR showed that switch toward STAT3b form is sustained in cells continuously treated with ST2 (Figure 5C). However, when MDA-MB-435s cells treated with ST2 for 4 days were placed in fresh media, the beta isoform gradually reverted to its initial level (Figure 5D).

Therefore, we were able to develop a tool to efficiently re-direct STAT3 exon 23 alternative splicing at the endogenous level. This effect is revertible and depends on the continuous presence of the morpholinos in the media.

Knocking down of STAT3 by morpholino

The described morpholinos allow us to induce the production of the STAT3b isoform while simultaneously reducing the level of the alpha isoform. To distinguish between the appearance of the beta isoform and the disappearance of the alpha one, we designed two additional morpholino oligonucleotides to generate a null STAT3 mutant using a modified splicing re-direction approach we have been developing that exploits Nonsense-Mediated Decay (NMD), which we called forced splicing–dependent NMD (FSD-NMD), where alteration of splicing introduces a Premature Termination Codon (PTC), which in turn triggers NMD. The two oligos (ST6 and ST7) were targeted to either splice sites of exon 6, which is 152nt long and therefore - when skipped- introduces a frameshift and an early stop codon (Figure 6A). This not only produces a short polypeptide lacking all the STAT3 functional domains, but the mRNA itself becomes highly destabilized by NMD, because of the PTC caused by the frameshift.



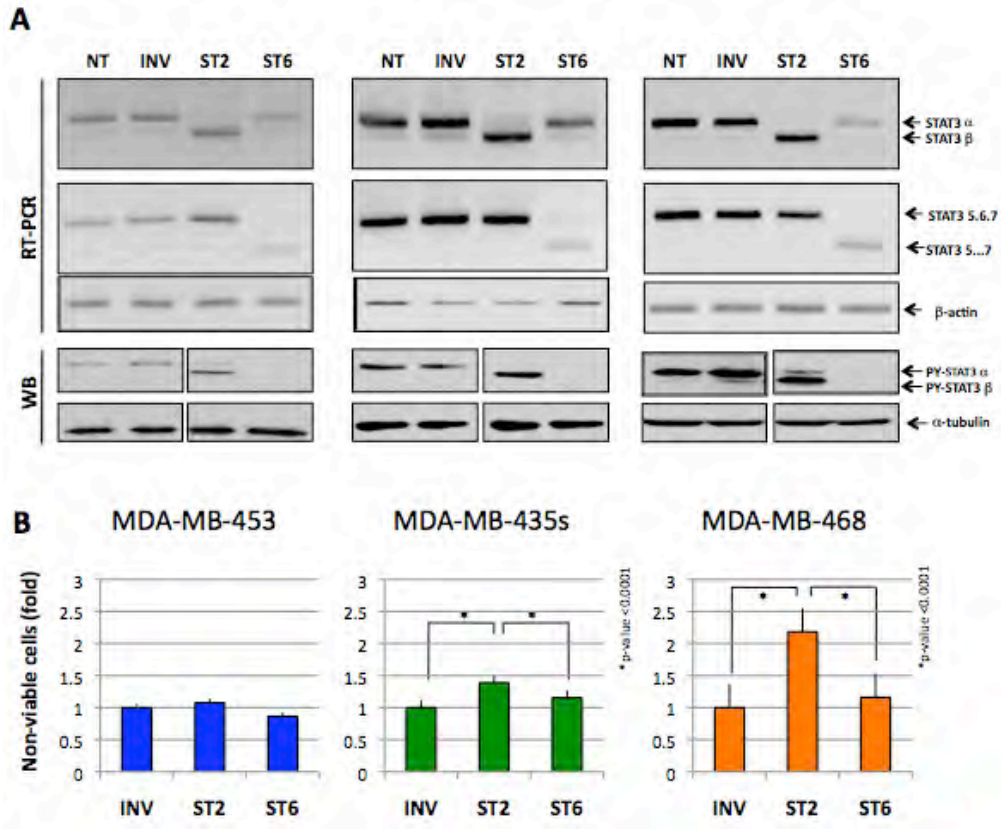
6. Total Knock-down of Stat3 by Forced Splice-Dependent NMD (FSD-NMD)

(A) Schematic representation of the strategy used for STAT3 knockdown. Morpholino oligos ST6 and ST7 cause skipping of exon 6 leading to a frameshift and a pre-mature termination codon (PTC), ultimately causing RNA degradation following nonsense mediated decay (NMD). **(B**) RT-PCR and Western blot analysis of STAT3 a/b levels in MDA-MB-435s cells treated with 16mM of ST6, ST7 or INV for 4 days. Tubulin was used as loading control.

Indeed, when we treated MDA-MB-435s cells with either the ST6 or the ST7 morpholinos for 4 days, each of them induced dramatic exon 6 skipping and a significant destabilization of the alternatively spliced mRNA, which was barely detectable by PCR. Similarly, full-length protein was virtually undetectable, with almost complete loss of both isoforms, as shown by Western blot (Figure 6B). Finally, to quantify the extent of the knockdown of both STAT3 isoforms achieved by the FSD-NMD strategy, we performed a side-by-side comparison with the more standard siRNA approach. As shown in Supplemental Figure 2, FSD-NMD is at least as efficient as standard RNAi approach, with the added advantage that its results can be directly compared and contrasted to splicing re-direction (ST2) without having to use two separate treatments, resulting in more properly controlled experiments. Both ST6 and ST7 resulted to be efficient tools to knockdown both STAT3 isoforms. The ST6 compound was chosen for all subsequent experiments since, as ST2, it can also target the mouse STAT3 sequence.

Effect of STAT3 alternative splicing modulation *in vitro*

STAT3b has been described as a dominant negative isoform able to interfere, when overexpressed, with STAT3 role in tumor growth and survival. We therefore decided to compare the effect of inducing STAT3b versus knocking down both isoforms on cell viability.



7. Effect of STAT3 alternative splicing modulation *in vitro*. (**A**) RT-PCR and Western blot analysis of STAT3 a/b levels in MDA-MB-453, MDA-MB-435s and MDA-MB-468 cells treated with 16mM of INV, ST2 or ST6 oligos for 4 days. Tubulin was the loading control for Western blot. (**B**) Cell death quantification (represented as fold change relative to control treatment) determined by trypan blue exclusion for MDA-MB-453, MDA-MB-435s and MDA-MB-468 cells treated with 16mM of INV, ST2 or ST6 oligos for 4 days.

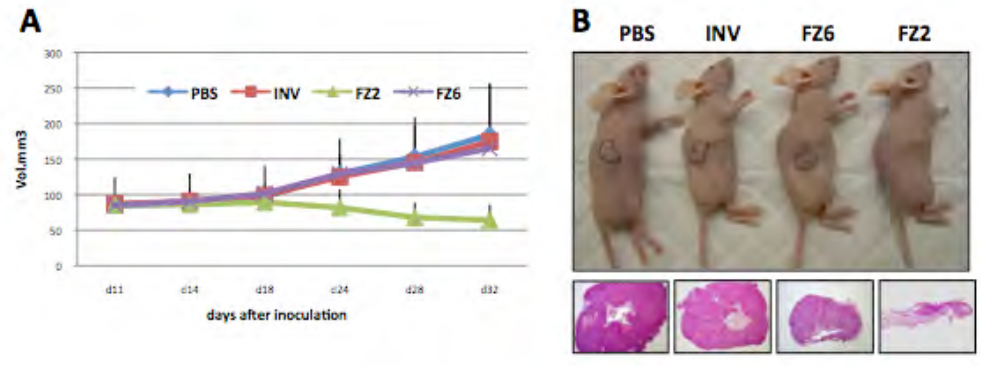
Thus we treated three breast cancer cell lines known to have different degree of addiction to STAT3 (MDA-MB-453<MDA-MB-435s<MDA-MB-468[53]), with either the ST2 or the ST6 morpholinos. As expected, cells treated with the ST2 compound showed an induction of STAT3b and a reduction of STAT3a, while cells treated with ST6 showed a reduction of both isoforms, as confirmed both at the RNA and protein levels (Figure 3C). After 4 days of treatment cell viability in both treatment groups was assayed. Induction of STAT3b in the ST2 treated cells caused a significant increase in cell death (Figure 3D) reflective of the degree of STAT3 addiction in the different cell lines, being more pronounced in MDA-MB-468 than MDA-MB-435s while being null in MDA-MB-453, which are not addicted to STAT3.

On the contrary, ablation of both STAT3 isoforms, obtained with the ST6 oligo, had a much less pronounced effect on cell viability in both MDA-MB-435s and MDA-MB-468 (Figure 7B).

Modulation of STAT3 alternative splicing *in vivo*

So far our data suggest that redirection of alternative splicing of the STAT3 gene can induce a decrease in cell viability *in vitro*. We then set out to test whether STAT3 alternative splicing modulation could affect tumor growth *in vivo* using morpholino oligonucleotides covalently linked to an octaguanidine dendrimer (vivo-morpholinos) [54].

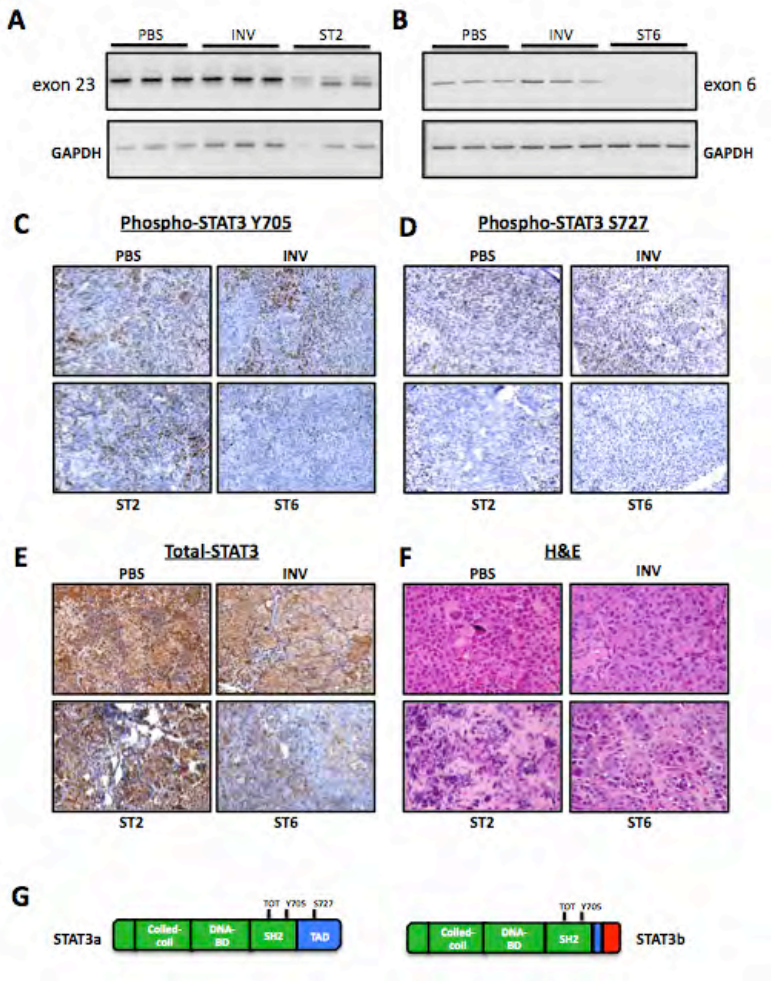
Since delivery of vivo-morpholino oligos in tumors hasn't been reported in the literature, as a first attempt we decided to deliver the vivo-morpholino oligos into tumors by either intra-venous (IV) or intra-tumor (IT) administration. Athymic female mice were subcutaneously implanted with MDA-MB-435s cells. When tumors reached an average of 100mm3 in volume size, mice (n=3/group) were randomly assigned to one of the 4 treatment groups (Control-IV, ST2-IV, Control-IT and ST2-IT). Animals were treated twice a week for one week and 4 days after the second treatment tumors were collected and further analyzed. To determine the efficacy of the treatments total RNA was extracted from the tumors and corresponding cDNAs were analyzed by PCR for the relative level of STAT3a and STAT3b isoforms. While intratumoral injection induced a clear



8. Effect of Stat3 splicing modulation in vivo. 10^{7} MDA-MB-435s cells were subcutaneously inoculated in the flanks of athymic mice. When tumors reached approximately 100 mm^3 in volume size, mice (n= 5/group) were randomly assigned to one of the 4 treatment groups (PBS, INV, FZ2 and FZ6). Vivo-morpholino oligos were intratumorally injected (0.12mg in a volume of 30ul) twice a week for a total of 3 weeks and tumor volumes were measured twice a week. Four days after the last treatment tumors were harvested **B**, (Top) Representative image of mice in each treatment group at time of sacrifice. (Bottom) Representative H&E staining of tumor sections (2X magnification)

switch from STAT3a to STAT3b in at least two of the tumors, intravenous administration didn't have any effect (Supplemental fig.3). However no histo-pathological changes or statistically significant difference in tumor size were found between control and intra-tumorally treated tumors (data not shown).

Next we decided to increase the length of the treatment and to include the ST6 oligo in order to get a better understanding of the STAT3 isoforms contribution to tumor growth. Athymic female mice were subcutaneously implanted with MDA-MB-435s cells. When tumors reached an average of 100mm3 in volume size, mice (n=5/group) were randomly assigned to one of the 4 treatment groups (PBS, INV, ST2 and ST6). Vivo-Morpholino oligos were intratumorally injected twice a week for a total of 3 weeks while monitoring for tumor growth. At the end of the treatment period tumors were collected and further analyzed. Interestingly, tumors treated with the ST2 oligo (inducing the switch from STAT3a to STAT3b) stopped growing right after the first treatments and started regressing (Figure 8A and 8B). Surprisingly, tumors treated with the ST6 oligo (which knocks down both STAT3 isoforms) grew apparently as well as the PBS and INV-treated tumors.



9. Effect of STAT3 alternative splicing modulation *in vivo.* **A**) RT-PCR analysis of STAT3 exon 23 levels in three tumors for each treatment group. GAPDH was used as housekeeping gene. **(B)** RT-PCR analysis of STAT3 exon 6 levels in three tumors for each treatment group. GAPDH was used as housekeeping gene.**(C, D and E)** Representative images of tumor sections subjected to IHC for phospho-STAT3 Y705, phospho-STAT3 S727 and tot-STAT3 respectively (40X magnification). **(F)** Representative images of tumor sections stained for H&E (40X magnification). (**G**) Epitopes map on Stat3 and Sta3beta

To verify the efficacy of the treatments *in vivo*, tumor samples were analyzed both at the RNA and protein level. RT-PCR for either STAT3 exon 23 (Figure 9A) or STAT3 exon 6 (Figure 9B) confirmed efficacy of both ST2 and ST6 treatments in inducing either STAT3 splicing switch or total STAT3 FSD-NMD knockdown. Tumor sections were subjected to immunohistochemistry (IHC) against three STAT3 antibodies: phospho-STAT3 Y705 (present in both STAT3a and STAT3b) didn't give any signal in ST6 treated tumors, confirming that the ST6 compound was effective at substantially knocking down STAT3 at the molecular level, whereas the level of phospho-STAT3 Y705 in ST2-treated tumors was comparable to control-treated tumors, consistently with the maintenance of this phosphorylation site in STAT3b (Figure (C). IHC for phospho-STAT3S727 (present only in STAT3a) and for total STAT3 were negative for both ST2 and ST6 treated tumors, further confirming the efficacy of the treatments at the protein level (Figure (9D and 9E).

Tumor sections were stained for H&E and scored for necrosis and mitotic count, showing that the only treatment associated with a significant effect in terms of tumor morphology is the splicing switch induced by the ST2 compound (Figure 9F). Histo-pathological examination of the ST2 treated tumors revealed that they had the lowest mitotic index, in agreement with the nearly absence of tumor material (Supplemental Figure 4A). In the same way, the fact that they had the lowest scoring for necrosis and apoptosis (Supplemental Figure 4B and 4C) could still reflect their extreme shrinking in size, limiting the amount of tumor surface area. Of particular interest is that while STAT3 knock-down was effective at the molecular level, it didn't seem to have major effects on tumor biology and growth, except for the appearance of more evident cytologic alterations compared to controls (Figure 9F).

Taken together these data demonstrate that alternative splicing re-direction in tumors is a feasible and powerful approach and that, in the case of STAT3, the induction of STAT3b *in vivo* has a much more dramatic effect than *in vitro*.

STAT3-beta unique transcriptional signature

Our data show that reduction of STAT3a and concomitant induction of STAT3b by alternative splicing modulation can decrease cancer cell viability more efficiently than the knockdown of both STAT3 isoforms. Therefore we set out to determine which STAT3 target genes may be involved in this process.

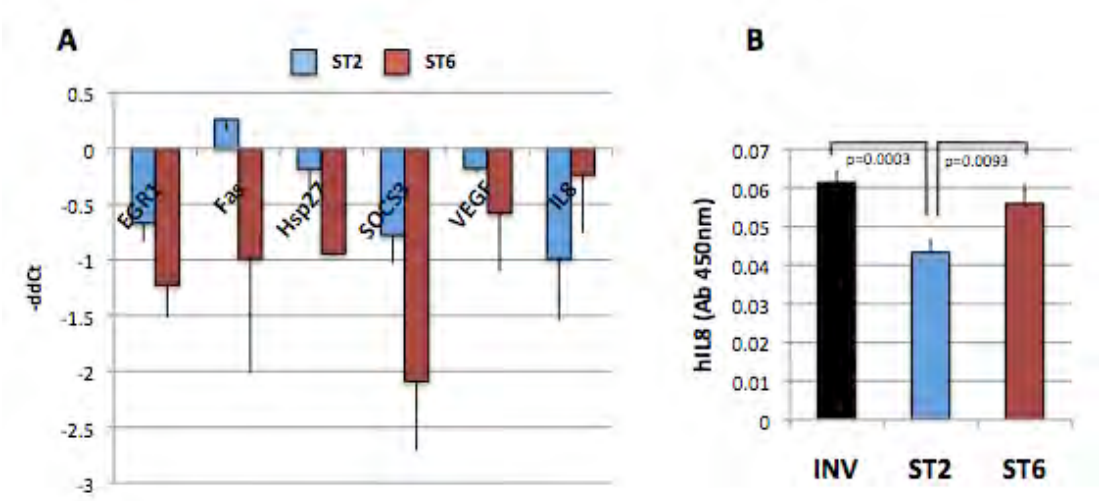
Based on the predominant promoter occupancy dominant-negative model, on the observed efficacy of the splicing re-direction treatment at the molecular level and biological effects of the treatments, we expected to see some general down-regulation of described STAT3 'canonical' targets with the knock-down and a more pronounced effect when the dominant negative isoform is induced by splicing re-direction (ST2).

cDNAs from MDA-MB-435s cells treated for 4 days with ST2 (STAT3b induction), ST6 (STAT3 K.O.) or INV (control) compounds were analyzed by real-time PCR. Whereas knocking-down both STAT3 isoforms by FSD-NMD using the ST6 compound resulted in a somewhat modest but consistent downregulation of most canonical targets analyzed (and a robust downregulation of STAT3 itself, as expected), STAT3b induction by

ST2 didn't act as a "dominant-negative" regulator of transcription at all (Figure 10A). Indeed, it appears that the expression of the canonical targets is almost unaffected, suggesting that their control by STAT3 relies on a mechanism independent from the C-terminal region of STAT3 usually described as the transactivation domain (which is absent in the beta isoform).

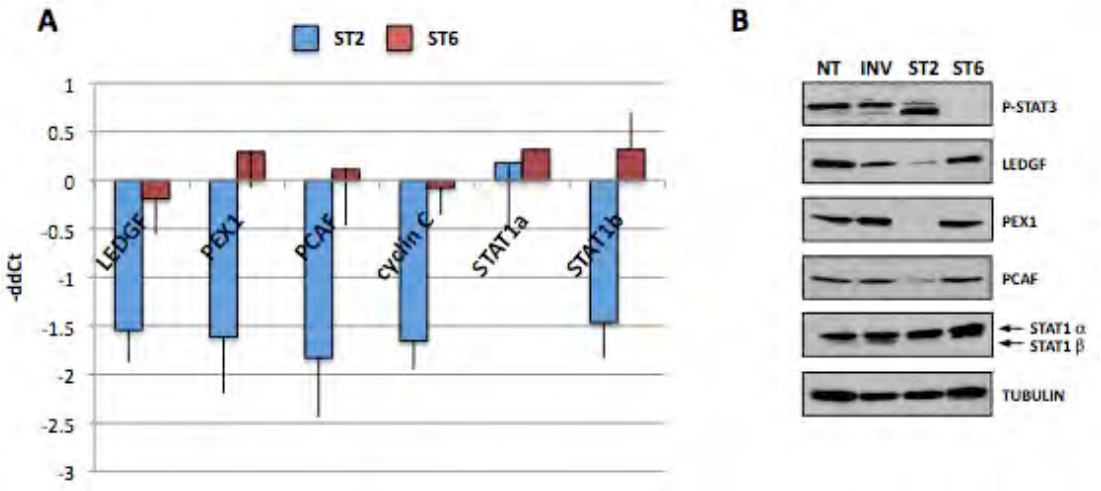
An exception was represented by interleukin 8 (IL8), which consistently behaves in the opposite way and was downregulated by the splicing switch but not by STAT3 total knockdown (Figure 10A). Specific down-regulation of IL8 by the splicing switch to STAT3b was also confirmed at the protein level by ELISA assay on media from MDA-MB-435s cells treated with ST2, ST6 or INV compounds for 4 days (Figure 10B).

Furthermore, the fact that IL8 behaved differently from other STAT3 'canonical' target genes upon ST2 treatment suggested us that there might be different classes of target genes in terms of how they respond to STAT3a/STAT3b regulation.



10. **STAT3beta switch vs. Stat3 knockdown effect on canonical Stat3 targets**. **(A)** cDNAs from MDA-MB-435s cells treated with 16mM of either ST2, ST6 or INV morpholinos for 4 days were analyzed by real-time PCR. Results were normalized to the housekeeping gene HRPT and are represented as -ddC(t). **(B)** IL8 quantification by ELISA assay on media from MDA-MB-435s cells treated with 16mM of either ST2, ST6 or INV morpholinos for 4 days.

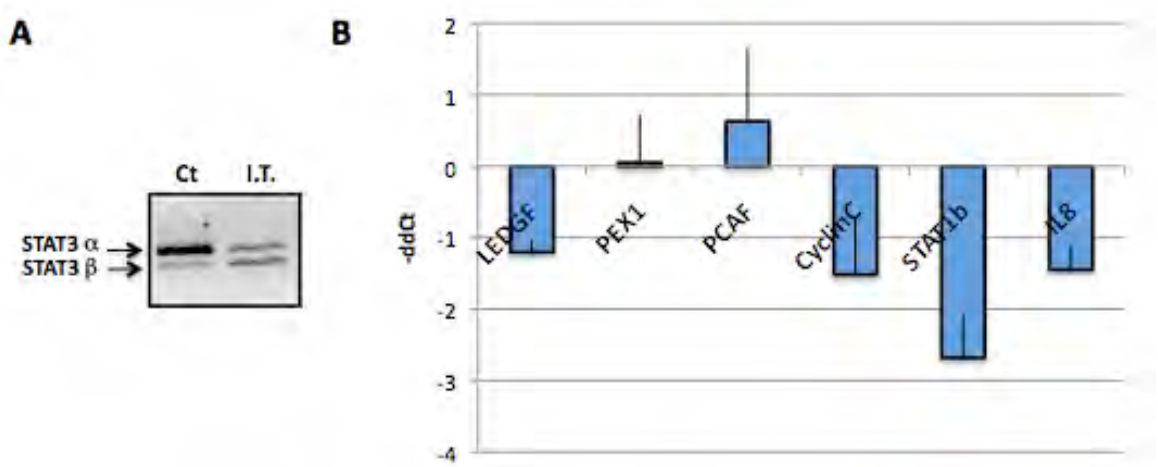
To investigate whether STAT3b might modulate additional new target genes, we compared ST2-treated or ST6-treated versus INV-treated MDA-MB-435s cells using the microarray platform U133A 2.0 (Affymetrix). Genes having a fold change ≥ 2 were considered to be significant (Supplemental figure 5), and were further validated by real-time PCR and/or Western blot (Figure 11A and B). Five novel genes were confirmed to be downregulated both at the RNA and protein level by the switch to STAT3b, but not by knockdown of both STAT3 alpha and beta: LEDGF (chromatin binding protein and transcriptional co-activator; a pro-survival and growth factor); PEX1 (member of the AAA ATPase subfamily, a peroxisomal protein required for import); cyclin C; PCAF (histone acetyltransferase and transcriptional co-activator, promotes growth, invasion and resistance); STAT1b (dominant negative isoform of STAT1). Notably, the microarray analysis confirmed that none of the canonical STAT3 targets, except for IL8, were downregulated by the



11. **STAT3b switch activation of specific target genes**. **(A**) cDNAs from MDA-MB-435s cells treated with 16mM of either ST2, ST6 or INV morpholinos for 4 days were analyzed by real-time PCR to validate STAT3b targets identified by microarray analysis. Results were normalized to the housekeeping gene HRPT and are represented as -ddC(t). **(B**) Lysates from MDA-MB-435s cells treated with 16mM of either ST2, ST6 or INV morpholinos for 4 days were analyzed by western blot to validate STAT3b targets identified by microarray analysis.

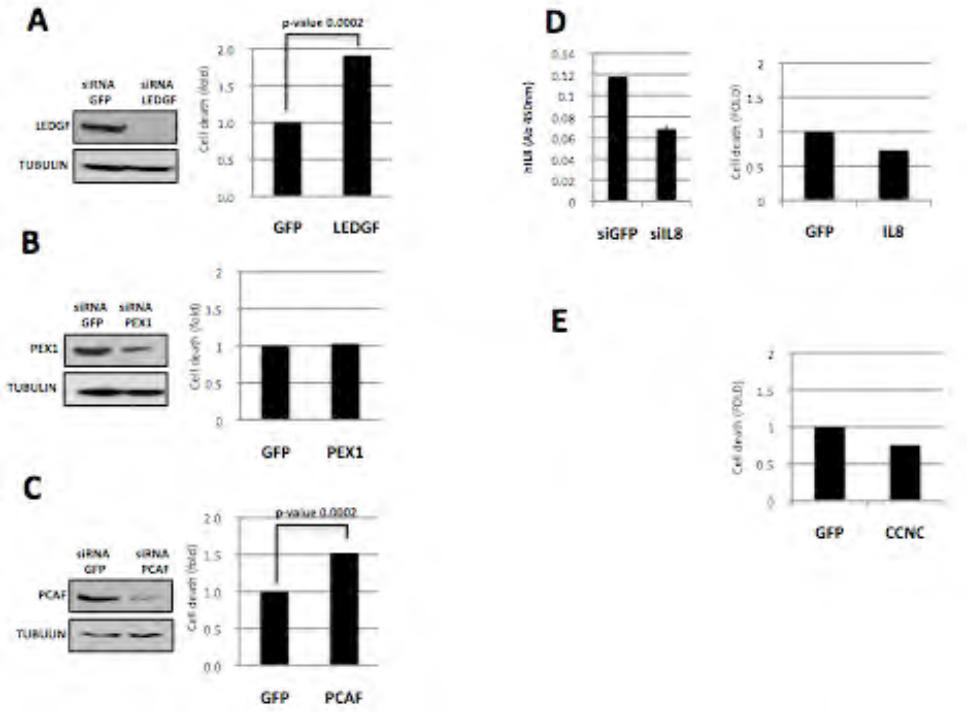
STAT3b induction, giving support to our hypothesis of STAT3β as an anti-tumorigenic isoform uncoupled from its proposed 'dominant negative' properties.

Next we wanted to investigate if the same genes specifically downregulated by the switch to STAT3b *in vitro* were also affected *in vivo* in the ST2-treated tumor samples. Real-time PCR was performed on the cDNAs f rom the tumors treated intra-tumorally with the ST2 compound for 1 week (because of the extreme shrinking in size we didn't have enough RNA to analyze for the tumors treated for 3 weeks) (Figure 12A). Although for both PEX1 and PCAF we couldn't measure a decrease in expression, for the other four target genes we were able to detect a significant downregulation of their levels, similar to what we observed *in vitro* (Figure 12B).



12. **STAT3b switch activation of specific target genes in tumors**. **(E)** (Left) RT-PCR analysis of STAT3 a/b levels in control (Ct) or ST2-treated (I.T.) xenograft tumors. (Right) Real-time PCR analysis of STAT3b target genes in ST2-treated tumors compared to control-treated tumors. Results were normalized to the housekeeping gene B2M and are represented as –ddC(t).

From these experiments we conclude that STAT3b doesn't behave as a dominant negative regulator of STAT3a, but instead in our system it modulates a specific set of targets genes both *in vitro* and *in vivo*.



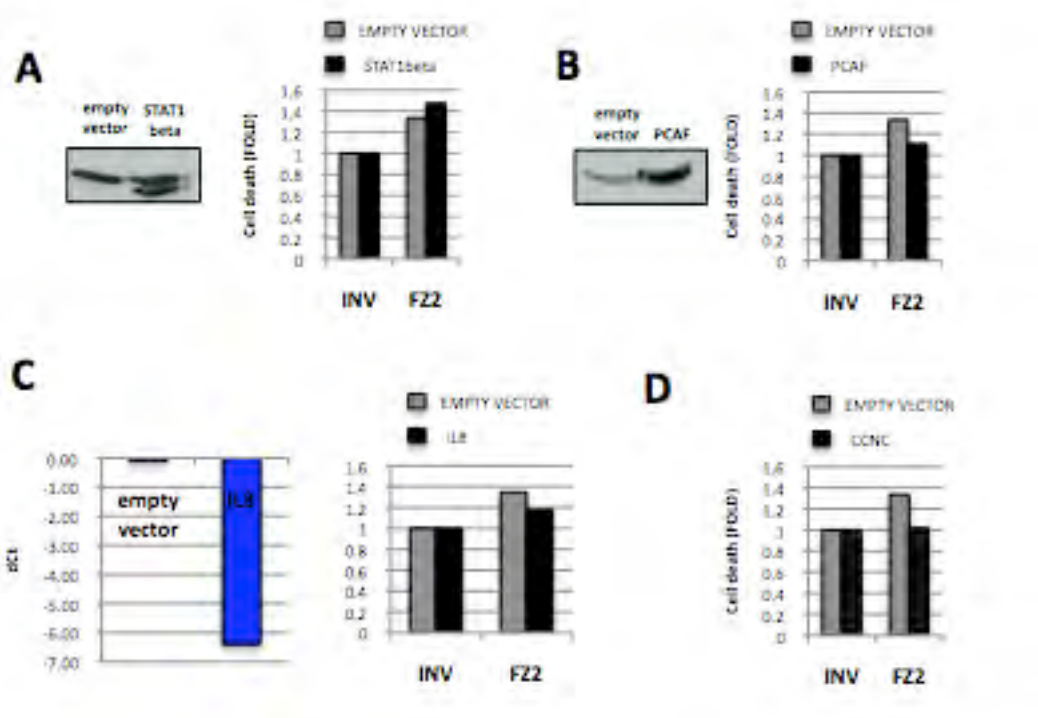
13. **Knockdown of STAT3b target genes**(A) (Left) Lysates from MDA-MB-435s treated with siRNA against GFP or LEDGF were immunoblotted using antibody against LEDGF. Tubulin was used as loading control. (Right) Cell death quantification (represented as fold change relative to control treatment) determined by trypan blue exclusion for MDA-MB-435s treated with siRNA against GFP or LEDGF. **(B to E)** Same as in (A) for PEX1, PCAF, IL8 and CCNC, respectively.

Knockdown and rescue of STAT3b targets

The set of target genes specifically downregulated by the switch from STAT3a to STAT3b has never been associated with STAT3beta functions. In order to get a better understanding of their contribution to the effect observed when STAT3 splicing is redirected from STAT3a to STAT3b in cancer cells we took two different approaches. First we were interested in testing if their downregulation was able to mediate a decrease in cell viability, as observed with the ST2 treatment. MDA-MD-435s cells were treated with siRNA against each of the five target genes (for STAT1 we couldn't use any siRNA oligos able to specifically target the beta isoform) and cell viability was measured. Whereas LEDGF and PCAF downregulation caused a significant increase in cell viability similar to what observed with the ST2 treatment (Figure 13A, 13C and 13C), no major effects were observed when PEX1, IL8 or CCNC were downregulated (Figure 13B, 13D and 13E). Next we wanted to investigate if forced expression of STAT3b target genes was able to rescue the effect on cell viability induced by the switch from STAT3a to STAT3b. MDA-MB-435s stably expressing each of the five STAT3b target genes were treated with either ST2 or INV compounds and cell viability was measured after 4 days of treatment

(we didn't pursue PEX1 since it is not downregulated in the treated tumors and its downregulation didn't have any effect on cell viability). With the exception of STAT1b, whose forced expression didn't affect the increase in cell death induced by the switch from STAT3a to STAT3b (Fig.6G), the other targets all significantly inhibited, although to a different extent, the induction of cell death caused by the ST2 treatment (Fig. 14A-D). Overexpression of LEDGF wasn't achieved due to difficulties in cloning its cDNA, but the experiment is ongoing.

Taken together these data suggest that the effect of the STAT3b isoform on cell viability is to some extent mediated by the downregulation of a specific set of target genes.



14. **Rescue of STAT3beta-dependent cell death by overexpression of STAT3beta downregulated genes** (A) Left) Lysates from MDA-MB-435s stably expressing STAT1beta or empty vector were immunoblotted using antibody against STAT1. Tubulin was used as loading control. (Right) Cell death quantification (represented as fold change relative to control treatment) determined by trypan blue exclusion for MDA-MB-435s stably expressing STAT1beta or empty vector treated with 16uM of INV or ST2 morpholino oligo for 4 days. **(B to D)** Same as in (A) for CCNC, PCAF and IL8, respectively.

KEY RESEARCH ACCOMPLISHMENTS

1. **Identification of splicing regulatory elements contolling Stat3 alternative splicing**
2. **Characterization of compounds that induce stat3 alternative splicing shift to the beta isoform *in vitro* and *in vivo***
3. **Development of FSD-NMD knock-down technology**
4. **Identifcation of Stat3beta-specific trascriptional targets**
5. **Identification of a new mechanism of action for Stat3beta**
6. **Identification of LEDGF and PCAF/300 as potential new targets in breast cancer therapy**
7. **Demonstration that modulation of a single splicing event can have potent anti-oncogenic activity in vivo**
8. **Induction of tumor regression in Stat3-dependent breast cancer models**

REPORTABLE OUTCOMES

Abstract/presentations

Zammarchi F. et al. **Redirection of STAT3 alternative splicing: a novel anti-tumoral approach**.

3nd annual postdoctoral research symposium, Memorial-Sloan Kettering Cancer Center, New York, U.S.A. (2009).

Zammarchi F. et al. **Modulation of STAT3 alternative splicing as a tool to dissect its anti-tumorigenic potential**.

RNA Society's 2010 Annual Meeting, Seattle, (2010).

Zammarchi F. et al. **Anti-tumorigenic potential of STAT3 alternative splicing modulation**.

4th annual postdoctoral research symposium, Memorial-Sloan Kettering Cancer Center, New York, U.S.A. (2010).

Cartegni L. **Therapeutic modulation of RNA processing**.

Presented at 24th International Symposium: "RNA Structure and Function: a new frontier in biomedical research" Hunter College, New York (2011).

A manuscript is in preparation for publication

CONCLUSIONS

STAT3 is an oncogene whose constitutive activation in almost 70% of solid and hematological tumors is thought to contribute to multiple aspects of the tumorigenesis process. Its main alternative splicing isoform, STAT3β, has been described as a dominant negative factor that, when overexpressed in cancer cells, is able to inhibit cell growth *in vitro* and *in vivo*. However, an increasing number of studies have revealed new biological properties of STAT3β, in addition and in alternative to its dominant negative functions.

The data presented in this work support this new vision of STAT3β and show how, by the modulation of its expression levels *via* splicing re-drection, it can behave as a potent anti-tumorigenic isoform trough the modulation of its own specific set of target genes.

Although STAT3b has been recognized as an essential player in the STAT3 biology for many years, nothing is know about its alternative splicing regulation. In this study **we have identified an essential regulatory Exonic Splicing Enhancer (ESE)** located at the 5' of exon 23 and designed a set of morpholino-based oligonucleotides, directed at blocking either the 3' splice site and/or the nearby enhancer. **These antisense compounds showed excellent re-direction capabilities of the endogenous STAT3 a/b isoforms**. The ability to modulate endogenously the relative levels of the STAT3a/STAT3b isoforms provided us with a power tool to elucidate STAT3b functions in a more physiological context thus avoiding artifactual results often associated with other techniques**. In addition, we designed morpholino oligos able to induce forced splicing–dependent NMD (FSD-NMD), a novel modified splicing re-direction approach that, by triggering NMD, led to destabilization of STAT3 mRNA**. This approach allowed us to accurately un-couple the effects due to the appearance of the beta isoform from those due to the simple down-regulation of the alpha isoform, using a single common technique. This is important because any gene-expression manipulation strategy induces some treatment-dependent, non-specific effects, therefore in this case such non-specific effects, if present will be common and can be dismissed when comparing knock-down to switching.

The data presented in this work highlight two important aspects: the first one is **that induction of the beta isoform had a much bigger effect compared to the knockdown of both isoforms**, both *in vitro* and particularly *in vivo*, where it caused a virtually complete regression of the tumors, in line with the early idea that STAT3β is a dominant negative factor. The second essential finding is that when we looked at the expression profile of a panel of STAT3 canonical target genes, none of them was significantly downregulated by the endogenous switch from STAT3a to STAT3b, as it would be expected if STAT3b was classical a dominant negative factor (and as it is observed when STAT3b is exogenously overexpressed at high levels, data not shown). The surprising lack of inhibition of the canonical STAT3 targets suggests that **the mechanism of action of STAT3b at physiological levels might be more complex than the suggested dominant negative effect** (which is mostly derived from high-level over-expression experiments). On the contrary, effective knockdown of both isoforms results in only a somewhat modest (although consistent) downregulation of most canonical targets analyzed, which further support the molecular efficacy of our FSD-NMD approach. These data suggest that under physiological conditions, the expression of these canonical targets is not dependent on the presence of the Trans-Activation Domain of STAT3 and most importantly **they strongly challenge the promoter-occupancy**

"dominant-negative" hypothesis commonly proposed for STAT3β, which instead might function, at least in certain contexts, through a completely separate mechanism.

Many reasons can be put forward to explain why the STAT3b effect *in vivo* is significantly more effective than what observed *in vitro*. The observation that STAT3 effect on cancer cell viability is less pronounced, if not opposite, *in vitro* than *in vivo* has been already described for STAT3 [27, 55] and it can be explained by context dependency and by its involvement in multiple aspects of the tumorigenesis process. Moreover, our morpholino oligos are homologous to the mouse sequence and they can re-direct STAT3 splicing in the surrounding murine cells. This could lead to STAT3a decrease and simultaneous STAT3b induction in the tumor microenvironment, including stromal and immune cells, thus potentially intensifying the effect of the STAT3 splicing switch *in vivo*.

Our study discovers a new set of target genes specifically downregulated, in our system, by the STAT3b isoform both *in vitro* and *in vivo*: LEDGF, PCAF, Cyclin C, PEX1, STAT1β and IL8 (the last one is the only described STAT3 target whose expression level is down-regulated by STAT3β). Among these targets LEDGF and PCAF are the ones able to induce cell death when downregulated and to inhibit STAT3b effect on cell viability when overexpressed. LEDGF (PSIP1) is a chromatin-associated cellular protein that has been implicated in transcriptional regulation, leukemogenesis, autoimmunity and HIV-integration [56-58]. Moreover, LEDGF has been described as a cancer associated survival protein able to stabilize lysosomes and to control a lysosomal cell death pathway rather than caspase-mediated apoptosis[59, 60]. Interestingly, a recent study showed an association between LEDGF overexpression and STAT3 activation via IL6 [61].

PCAF (p300/CBP associated factor) has an established role in regulating gene transcription by acetylating histones and its activity/specificity is in part modulated by the interaction with multiprotein complexes [62]. Given its contribution to different diseases, including cancer and asthma, PCAF has been recently identified as a potential drug target[63]. It is interesting to note that both LEDGF and PCAF are involved in transcriptional regulation and their inhibition could somehow explain the overall decrease in transcriptional activity observed in the ST2 treated cells.

One puzzling aspect emerging from our data is the modest effect of the ST6 oligo *in vitro* and the complete absence *in vivo*. One possible explanation for the *in vivo* results could be the moderate level of phospho-STAT3 addiction of the MDA-MB-435s cell line. Unfortunately we couldn't efficiently deliver our vivo-oligo in the more phospho-STAT3 addicted MDA-MB-468 cell line without non-specific toxic effects, but it is reasonable to think that the effect of downregulating STAT3 would have been higher in that context. Our *in vivo* results showing no effect when STAT3 is downregulated in tumors both at the RNA and protein level are in apparent disagreement with previous reports. However it is important to note that our approach target specifically STAT3 mRNA and hence is highly specific while other studies inhibit upstream activating kinases, like JAK2, affecting STAT3 in an indirect way and so increasing the probability of broader effects.

The ability to modulate STAT3β levels at physiological levels allowed us to redefine its contribution as an anti-cancer target without incurring in the common non-physiological artifacts often associated with cDNA overexpression. Indeed, our data uncovers a **novel mechanism of action for STAT3β, mediated by the downregulation of previously unidentified target genes.** This also implies that the commonly proposed promoter-occupancy dominant-negative mechanism is likely not correct for STAT3β under physiological conditions (although it might still hold true when overexpression of the beta isoform is involved). Finally, our results are in agreement with

recent studies showing that STAT3β might contribute to the activation of selective target genes and perform unique biological functions. Notably these studies have been conducted in mice where STAT3b has been genetically ablated and not with overexpression experiments [38, 39].

Use of antisense compounds is a well-established approach for gene expression regulation, and it normally relies on the destabilization of the target mRNAs, either by triggering RNAse H degradation or by taking advantage of the RNAi machinery. Redirection of alternative splicing differs from the standard antisense-based technologies in that the objective is not the degradation of the target RNA. On the contrary, the integrity of transcripts must be preserved and re-directed toward the desired outcome. The possibility to knock down the desired target, while at the same time inducing the expression of a favorable isoform represents probably the most relevant advantage of the splicing redirection approach. This in fact ensures that even a moderate efficacy at the targeting level translates into high treatment effectiveness and might in part account for the increased difference in performance of the two treatments from *in vitro* to *in vivo*. Specific and persistent splicing switch has been obtained *in vivo* in multiple model systems [64, 65], and splicing redirection compounds have entered clinical trials to treat Duchenne Muscular Dystrophy[66] and other diseases.

Our work shows for the first time how the modulation of a single endogenous splicing event in tumors can have powerful anti-tumorigenic capabilities and lead to tumor regression. Moreover, because of the intrinsic plasticity of this approach, the same strategy can be used to induce skipping of others STAT3 exons (coding for other domains such as DNA binding domain and SH2 dimerization domain) allowing the dissection of the different domains contribution to the many functions of STAT3.

In summary, the development of means to manipulate endogenous STAT3 alternative splicing in a physiological context allowed us to validate STAT3β as a potent anti-tumorigenic isoform, which works by a non-standard dominant negative effects and through a mechanism that involves targets that are not those typically associated to STAT3 activity. The flexibility and efficiency of this strategy makes it readily translatable to any other cancer genes with splicing isoforms involved in different aspects of tumorigenesis and opens new and exciting therapeutic perspectives.

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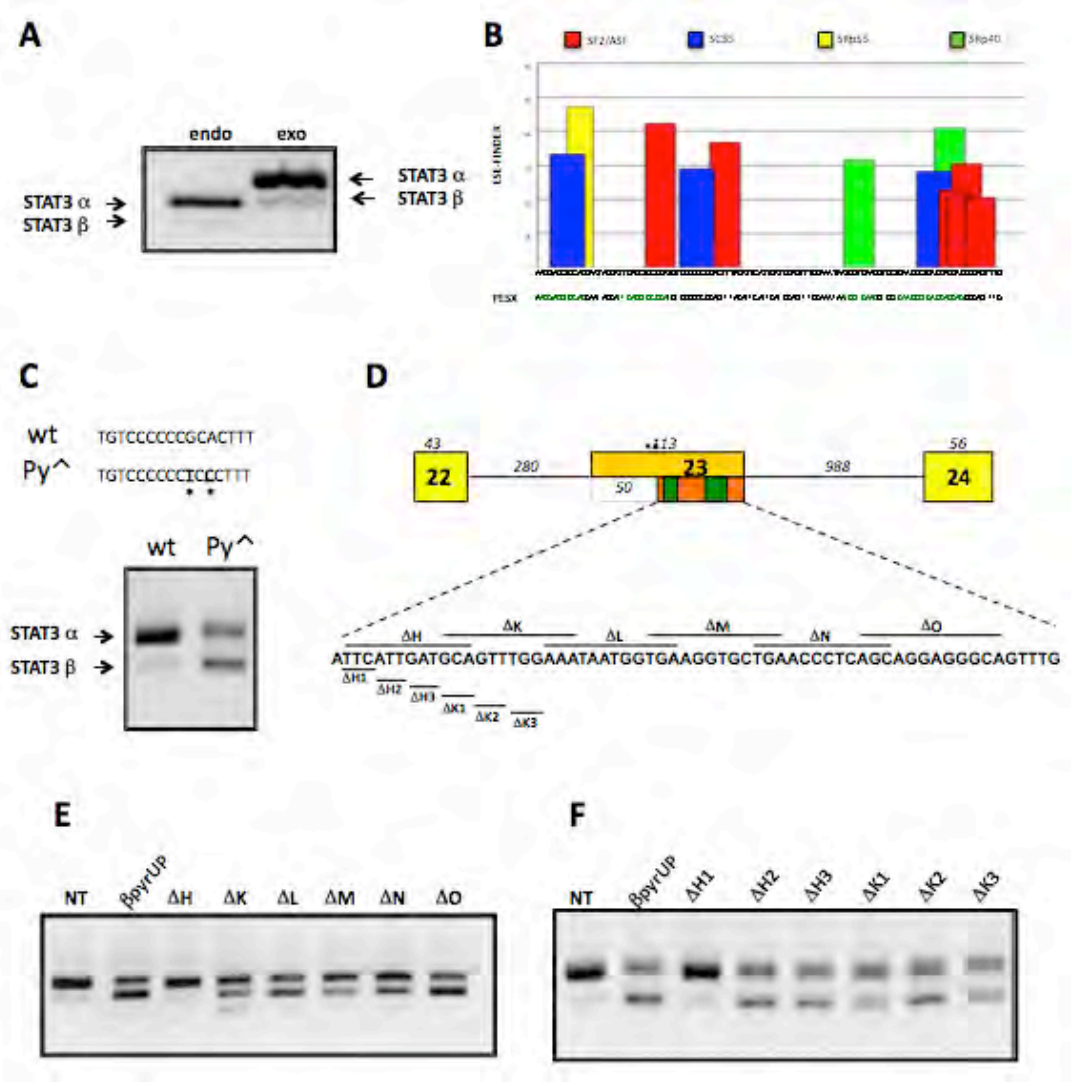
67. Dussault, A.A. and M. Pouliot, *Rapid and simple comparison of messenger RNA levels using real-time PCR.* Biol Proced Online, 2006. **8**: p. 1-10.

APPENDICES

SUPPORTING DATA



Supplemental figure 1

(A) RT-PCR analysis to assess the splicing pattern of endogenous STAT3 and transfected minigene in HeLa cells. Endo, endogenous; exo, exogenous.

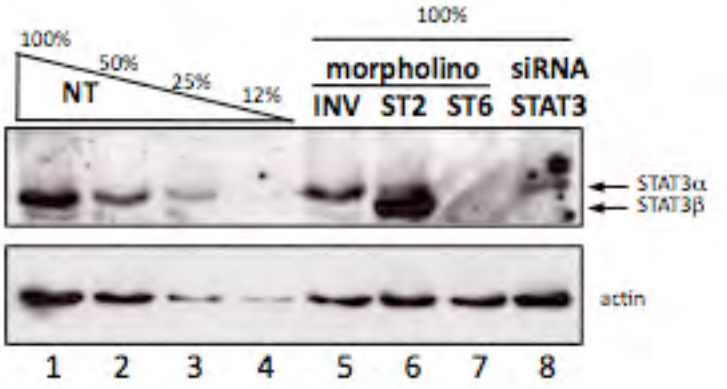
(B) Bioinformatic analysis using ESEfinder and PESX algorithms, aligned to exon 23 STAT3 sequence. The graph displays the predicted binding sites for 4 common SR proteins (SF2/AS, SC35, SRp55, SRp40) while in the PESX analysis green sequences represent putative exonic splicing enhancers.

(C) (Top) Partial sequence of the wild-type (wt) and pyrimidine enhanced (Py$^{\circ}$) minigenes. Asterisks indicate the exact position of the mutations. (Bottom) RT-PCR analysis of STAT3 α/β levels in both transfected minigenes in HeLa cells.

(D) (Top) Diagram of STAT3 minigene. Exon and intron sizes are indicated (nt). (Bottom) Lines above and below exon 23 sequence indicate the exact position of the 12nt (ΔH to ΔO) and 3nt (ΔH1 to ΔK3) deletions generated.

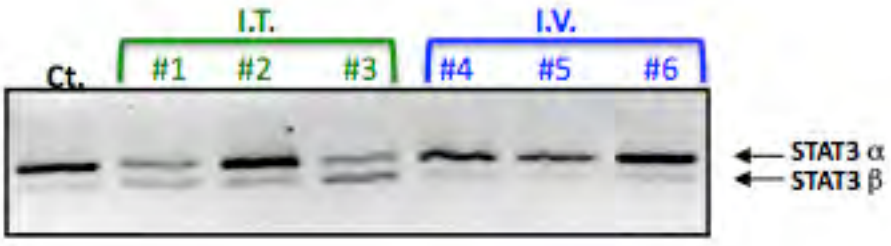
(E, **F)** RT-PCR analysis of STAT3 α/β levels in HeLa cells transfected with the 12nt (ΔH to ΔO) and the 3nt (ΔH1 to ΔK3), respectively, exon 23 deletion mutants. NT, untreated.

Supplemental figure 2



(A) Western blot of phospho-STAT3 Y705 levels in lysates from treated MDA-MB-435s. Lane 1 to 4 is a serial dilution of untreated cells (NT) from 100% to 12.5% of input. Lane 5 to lane 7 is 100% of input from cells treated with 16uM of the INV/ST2/ST6 oligos. Lane 8 is 100% of input from cells treated with 100nM of siRNA oligo against STAT3. Actin was used as loading control.

Supplemental figure 3

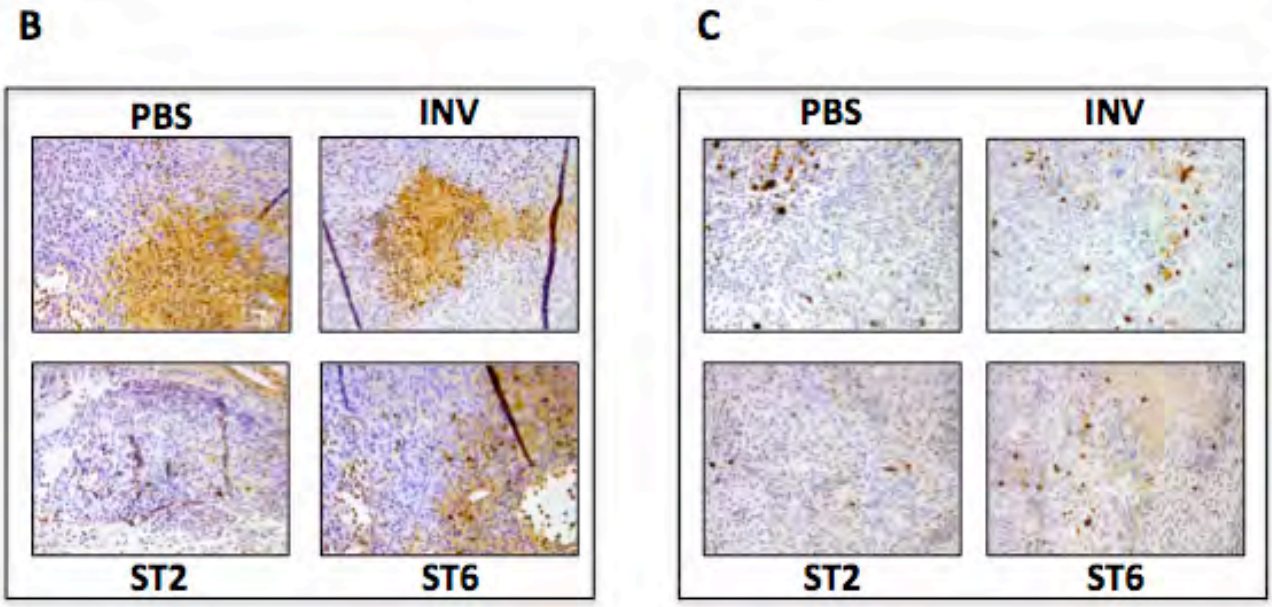


(A) RT-PCR analysis of STAT3a/b relative levels in intra-tumorally or intra-venously treated xenograft tumors twice a week for 1 week. Ct, control.

Supplemental figure 4

A

Group	Size (cm)	Necrosis (%)	Mitotic Count
Control			
mean	0.5	12	43
median	0.4	15	53
range	0.4-0.7	0-20	22-55
INV			
mean	0.4	20	137
median	0.45	20	146
range	0.4-0.45	n/a	101-163
ST2			
mean	0.175	2.5	8
median	0.175	2.5	8
range	0.1-0.25	0-5	1-15
ST6			
mean	0.48	23	68
median	0.45	20	75
range	0.43-0.55	5-45	19-109



(A) Table summarizing histo-pathological examination of treated tumors.

(B) Representative results of TUNEL staining for tumor sections from each of the four groups (40X magnification).

(C) Representative results of caspase3 immunohistochemical staining for tumor sections from each of the four groups (40X magnification).

A. ST2 compared to INV

Gene Name	Fold Change	Genbank	Gene Symbol	Description
218972_at	3,667	NM_018259	TTC17	tetratricopeptide repeat domain 17
204565_at	2,614	NM_018473	THEM2	thioesterase superfamily member 2
218104_at	2,579	NM_017746	TEX10	testis expressed sequence 10
201157_s_at	2,547	AF020500	NMT1	N-myristoyltransferase 1
201158_at	2,484	AI570834	NMT1	N-myristoyltransferase 1
220264_s_at	2,326	NM_020960	GPR107	G protein-coupled receptor 107
204809_at	2,309	NM_006660	CLPX	ClpX caseinolytic peptidase X homolog (E. coli)
214358_at	2,288	AW188201	ACACA	acetyl-Coenzyme A carboxylase alpha
209422_at	2,166	AY027523	PHF20	PHD finger protein 20
211506_s_at	0.343055556	AF043337	IL8	interleukin 8
201278_at	0.335416667	N21202	DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)
222118_at	0.315972222	AK023669	BM039	uncharacterized bone marrow protein BM039
203845_at	0.30625	AV727449	PCAF	p300/CBP-associated factor
222156_x_at	0.286111111	AK022459	CCPG1	cell cycle progression 1
203167_at	0.25625	NM_003255	TIMP2	TIMP metallopeptidase inhibitor 2
209337_at	0.23125	AF063020	PSIP1	PC4 and SFRS1 interacting protein 1
201955_at	0.217361111	AL137784	CCNC	cyclin C
205961_s_at	0.193055556	NM_004682	PSIP1	PC4 and SFRS1 interacting protein 1
209969_s_at	0.184027778	BC002704	STAT1	signal transducer and activator of transcription 1, 91kDa
204873_at	0.132638889	NM_000466	PEX1	peroxisome biogenesis factor 1

B. ST6 compared to INV

Gene Name	Fold Change	Genbank	Gene Symbol	Description
218972_at	4,348	NM_018259	TTC17	tetratricopeptide repeat domain 17
218104_at	2,573	NM_017746	TEX10	testis expressed sequence 10
204809_at	2:05	NM_006660	CLPX	ClpX caseinolytic peptidase X homolog (E. coli)
201157_s_at	2,391	AF020500	NMT1	N-myristoyltransferase 1
201158_at	2,388	AI570834	NMT1	N-myristoyltransferase 1
209422_at	2,221	AY027523	PHF20	PHD finger protein 20
204565_at	2,164	NM_018473	THEM2	thioesterase superfamily member 2
203167_at	0.308333333	NM_003255	TIMP2	TIMP metallopeptidase inhibitor 2
203973_s_at	0.296527778	M83667	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta
202238_s_at	0.277083333	NM_006169	NNMT	nicotinamide N-methyltransferase
202237_at	0.275	NM_006169	NNMT	nicotinamide N-methyltransferase
208992_s_at	0:15	BC000627	STAT3	signal transducer and activator of transcription 3
208991_at	0.071527778	BC000627	STAT3	signal transducer and activator of transcription 3

Supplemental Figure 5 (A) Table summarizing significant changes in gene expression in MDA-MB-435s cells obtained comparing levels of trascription measured using the microarray platform U133A 2.0 (Affymetrix) after treatment with ST2 compound (switch to STAT3beta) compared to control (INV) compound. Genes having a statistical significant ($p<0.05$) fold change of ≥ 2 in either direction are reported. **(B)** Table summarizing significant changes in gene expression in MDA-MB-435s cells obtained comparing levels of trascription measured using the microarray platform U133A 2.0 (Affymetrix) after treatment with ST6 compound (STAT3 knockdown) compared to control (INV) compound. Genes having a statistical significant ($p<0.05$) fold change of ≥ 2 in either direction are reported .